# Specific amino acids inhibit food intake via the area postrema or vagal afferents

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Abstract: To maintain nutrient homeostasis the central nervous system integrates signals that promote or inhibit eating. The supply of vital amino acids is tuned by adjusting food intake according to its dietary protein content. We hypothesized that this effect is based on the sensing of individual amino acids as a signal to control food intake. Here, we show that food intake was most potently reduced by oral L-arginine (Arg), L-lysine (Lys) and L-glutamic acid (Glu) compared to all other 17 proteogenic amino acids in rats. These three amino acids induced neuronal activity in the area postrema and the nucleus of the solitary tract. Surgical lesion of the area postrema abolished the anorectic response to Arg and Glu, whereas vagal afferent lesion prevented the response to Lys. These three amino acids also provoked gastric distension by differentially altering gastric secretion and/or emptying. Importantly, these peripheral mechanical vagal stimuli were dissociated from the amino acids' effect on food intake. Thus, Arg, Lys and Glu had a selective impact on food processing and intake suggesting them as direct sensory input to assess dietary protein content and quality in vivo. Overall, this study reveals novel amino acid specific mechanisms for the control of food intake and of gastrointestinal function.

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**Title:** Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents

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**E) Table of Contents:** Integrative

**Key Point Summary:**

- Proteins are more satiating than fats or lipids. Proteins are built by the 20 proteogenic amino acids.
- Here, we identified L-arginine, L-lysine and L-glutamic acid as the most potent anorectic amino acids in rats.
- L-arginine and L-glutamic acid require intact neurons in the area postrema to inhibit food intake, whereas L-lysine intact afferents fibers of the vagus nerve. All three mediate their effect by the blood stream.
- All three amino acids induce gastric distension by delaying gastric emptying and inducing secretion. However, the gastric phenotype does not mediate the anorectic response.
- These results unravel amino acid-specific mechanisms regulating digestion and eating behavior and thereby contribute to the understanding of nutrient sensing *in vivo*.

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**Abstract:**

To maintain nutrient homeostasis the central nervous system integrates signals that promote or inhibit eating. The supply of vital amino acids is tuned by adjusting food intake according to its dietary protein content. We hypothesized that this effect is based on the sensing of individual amino acids as a signal to control food intake. Here, we show that food intake was most potently reduced by oral Arg, Lys and Glu compared to all other 17 proteogenic amino acids in rats. These three amino acids induced neuronal activity in the blood-sensing area postrema and the nucleus of the solitary tract. Surgical lesion of the area postrema abolished the anorectic response to Arg and Glu, whereas vagal afferent lesion prevented the response to Lys. These three amino acids also provoked gastric distension by differentially altering gastric secretion and/or emptying. Importantly, these peripheral mechanical vagal stimuli were dissociated from the amino acids' effect on food intake. Thus, Arg, Lys and Glu had a selective impact on food processing and intake suggesting them as direct sensory input to assess dietary protein content and quality *in vivo*. Overall, this study reveals novel amino acid specific mechanisms for the control of food intake and of gastrointestinal function.

**Abbreviation List:**

4V, 4th ventricle; Ala, L-alanine; AP, area postrema; Arg, L-arginine; CCK, Cholecystokinin; DMX, dorsal motor nucleus vagus nerve; GLP1, Glucagon-like Peptide 1; Glu, L-glutamic Acid; GR, gracile nucleus; HK, high L-lysine dose (6.7 mmol/kg Lys); IG, intragastric; IP, intraperitonal; IV, intravenous; Leu, L-leucine; LK, low L-lysine dose (2 mmol/kg Lys); Lys, L-lysine; mTOR, mamaliane target of rapamycin; NTS, nucleus of the solitary tract; Phe, L-phenylalanine; PYY, Peptide YY; RLA, ringer lactate; Trp, L-tryptophane; Tyr, L-tyrosine.

**Introduction:**

Survival of an organism depends on its capability to adjust eating to its nutritional requirements. The brain ultimately controls this balance by integrating diverse signals related to the nutritional status of the body and the ingested meal both in terms of its energy- and macronutrient content (Morton et al. 2006). Meal born signals can be sensed centrally in specialized brain areas or be relayed from the periphery by abdominal vagal afferents, all finally leading to meal termination. The induction of these satiating signals might be triggered directly by circulating nutrients, indirectly by hormones released from enteroendocrine cells or via mechanical stimuli such as gastric distension as discussed in several reviews (Morton et al. 2006; Woods, 2009; Fromentin et al. 2012). In many cases, these mechanisms were studied for their role in energy homeostasis, whereas their macronutrient specificity was subject to less extensive research.

Protein intake is a necessity to supply the organism with essential amino acids and species as diverse as insects, fish, rodents, and humans adjust food intake according to its dietary protein content (Morrison et al. 2012; Anderson et al. 2004). The sensory mechanisms eliciting this behavior are however debated. Taste, smell, sight and texture of a meal have been suggested to help identifying its protein content based on learned associations, but experimental alterations of these cues did not prevent the anorectic effect of a high protein diet in rodents (L'Heureux-Bouron et al. 2004). Physiological sensory mechanism detecting the protein content of a meal might therefore originate from the gut or be post-absorptive for instance in peripheral nerves or directly in the brain. Indeed, the digestion of proteins to amino acids in the gastrointestinal tract stimulates vagal and endocrinal signals including CCK, GLP-1 and PYY release (Tome et al. 2009; Blom et al. 2006). However, total vagotomy did not interfere with the anorectic effect of a high protein diet (L'Heureux-Bouron et al. 2003) and the specificity of gastrointestinal hormones

for protein intake is debatable as lipids and carbohydrates induce similar hormonal responses (Morrison et al. 2012). An alternative possibility is that amino acids may act directly on nutrient sensing neurons. Indeed, it was shown that the injection of the amino acid Leucine directly into the brain ventricles inhibits food intake via modulation of mTOR-signaling (Cota et al. 2006; Blouet & Schwartz, 2012; Morrison et al. 2007). However, the role of this effect was challenged by the observation that physiological changes in plasma Leu concentration did not inhibit food intake even though circulating amino acids have access to the brain (Laviano et al. 2006; Zhang et al. 2007; Purpera et al. 2012; Nassl et al. 2011; Potier et al. 2009; Morrison et al. 2012). Importantly, all other 19 individual amino acids were so far not studied. Hence, the sensory mechanisms tuning ingestion of food to the dietary protein content remains elusive.

Here, we hypothesize that specific individual amino acids act as a physiological signal to control food intake. First, we systematically investigated which individual amino acid given by oral gavage had a specific impact on food intake in rats. We showed that Arg, Lys, and Glu were significantly more active than all other proteogenic amino acids at an isomolar dose. To localize their respective sensory mechanism, we performed surgical invalidations that demonstrated in the case of Arg and Glu the involvement of area postrema neurons, and in the case of Lys of vagal afferents, respectively. Interestingly, in the gut all three amino acids induced gastric distension by specifically inducing secretion or delaying gastric emptying. However, these peripheral mechanical stimuli were not linked to the control of food intake as intravenously applied Arg and Lys inhibited food intake, but did not modulate gastrointestinal function. These results demonstrate for the first time the importance of Arg, Lys and Glu for the independent adaptation of digestive functions and eating behavior probably as a direct sensory input to evaluate dietary protein content and quality.

## Methods:

*Ethical Approval*

All procedures for rat handling and experimental interventions were according to the Swiss Animal Welfare laws,approved by the Kantonale Veterinäramt Zürich and conform to the principles of UK regulations.

*Animal care*

Male Wistar rats (Janvier, France) were individually housed in wire-mesh hanging cages (room temperature 21 ± 1° C, artificial 12/12 h light dark cycle, water ad libitum, rat chow-3430 Kliba Nafag, Kaiseraugst, Switzerland).

*Application and euthanasia*

Intragastrically administered L-amino acids (freshly prepared, Sigma-Aldrich, Buchs, Switzerland) were applied in 2 ml tap-water. 6.7 mmol/kg was selected as a single dose for the screen based on comparison to other previous publications (Ng and Anderson, 1992; Bialik et al. 1989). The plasma amino acid changes measured after individual amino acid administration (S. Tab. 2) are in the range observed after the intake of a high protein diet (Peters & Harper, 1987).Intravenously administered L-amino acids were dissolved in Ringer-lactate and adjusted to pH 7.2. Animals were euthanized using pentobarbital (IP, 100 mg/kg, Kantonsapotheke Zürich, Switzerland) in combination with isoflurane (5 %) for quicker induction.

*Measurement of food and water intake*

Food/water intake was measured manually by weighing food containers/water bottles or using an automated system (BioDAQ, Research Diets, New Brunswick, USA). A meal was defined by the intake of at least 0.25 g chow, and the intermeal interval criterion was set to 10 min.

*Blood analysis*

Blood was collected into EDTA-coated tubes (Sarsted, Sevelen, Switzerland), inverted and centrifuged (1000 g, 10 min, 4 °C). Plasma amino acid concentration were measured by the Functional Genomics Center Zurich using high pressure liquid chromatography, plasma ions by enzymatic detection with Picollo Renal Functional Panel (Abaxis, Darmstadt, Germany) and plasma glucose by AccuCheck Aviva (Roche, Basel, Switzerland). Gastric secretion was previously shown to correlate with base excess in blood, an effect termed alkaline tide (S J Rune, 1966; Niv et al. 1993).

*Phenol red quantification*

Phenol red (Sigma) was quantified as described by others (Tsurugizawa et al. 2009). Phenol red recovery was 57.5 ± 0.6 % (Fig. S3A), respectively 74.3 ± 0.9 % (Fig. S3B) of the administered oral dose with no significant differences between treatment groups, thereby enabling comparison.

*cFOS immunohistochemistry*

This time point after gavage was chosen, because cFOS expression is strongest 90-120 min post-stimulation(Watts et al. 2006). Animals were anesthetized with pentobarbital (no isoflurane) and perfused transcardially with ice-cold phosphate buffer (0.1 M PB), followed by 4 % paraformaldehyde in PB. After removal brains were kept in paraformaldehyde for 2 h to achieve proper tissue fixation. Following incubation in 20 % sucrose solution (in PB, 48 h, 4°C) brains were snap frozen in hexane. Coronal sections (20 µm) were cut in a cryostat (CM 3050 Leica, Germany) throughout the brain. Every slice was thaw mounted on microscopic glass slides (SuperFrost Plus Faust, Switzerland). For the detection of cFos expression, frozen sections were air-dried at room temperature for 1 h and rehydrated in PBS. Unspecific binding was blocked by

2 h incubation in 1.5 % normal donkey serum. The primary antibody (1:5000, rabbit anti cFos, Ab-5, Calbiochem) was applied for 48 h at 4 °C. Sections were incubated with the secondary antibody (1:10'000, biotinylated donkey anti rabbit, Jackson 711-065-152) for 2 h at room temperature. After incubation in ABC (Vectastain-Elite ABC Kit, Vector Laboratories), followed by 0.05 % DAB solution (in 0.05 M Tris-HCl with 0.009 % $H_2O_2$ and color enhancement with 0.04 % $NiCl_2*6H_2O$ and 0.08 % $CoCl_2*6H_2O$), the sections were dehydrated in graded alcohols, cleared in xylene and fixed with entellan. The rat brain atlas of Swanson was used to localize the cFOS expressing neurons (Swanson, 2004). In 3 adjacent sections cFos positive cells were counted manually ca. at Bregma -13.76 in the AP and the NTS by a treatment blinded investigator.

*AP-lesion surgery*

AP-lesion was conducted similar to a thermal lesion approach described previously, except that the AP was removed by vacuum aspiration to reduce damage in the NTS (Lutz et al. 1998). Surgery success and specificity was verified functionally and histologically. The functional test was the absence of amylin induced satiation, which depends on an intact AP, and the presence of CCK induced satiation, which depends on intact vagal afferents (Lutz et al. 1998; Ruttimann et al. 2009).16 h food deprived rats were IP injected with amylin (10 µg/kg, Bachem AG, Bubendorf, Switzerland), CCK-8 (4 µg/kg, Bachem AG) or saline, respectively in a cross over design. AP-lesioned animals that did not reduce 30 min post-CCK application food intake by at least 30% were excluded due to a damaged vagus nerve (6 of 24 animals; food intake: sham NaCl 4.2 ± 0.3 g, sham CCK 2.1 ± 0.4 g, AP-lesion NaCl 2.9 ± 0.3 g, AP lesion CCK 0.9 ± 0.3 g). AP-lesioned animals that reduced 2 h post-amylin application food intake by more than 15% were excluded due to incomplete AP-lesion (7 of 24 animals; food intake: sham NaCl 8.6 ± 0.6

g, sham amylin 6.5 ± 0.5 g, AP-lesion NaCl 7.6 ± 0.6 g, AP lesion amylin 8.3 ± 0.7 g). Histological validation of the surgery included a test for the anterograde labeling capacity of vagal afferents to the NTS (Ruttimann et al. 2009). Rats were IP injected with 1 mg fluorogold (Fluorochrome, Denver, USA) in 1 ml saline 3 days before their brains were excised and fixed by a pH-switch protocol described by Khan et al (Khan et al. 2007). Brain sections were cut as described above, slides air dried and fixed with glycerol. An observer blind to surgery quantified the extent of the AP-lesion and counted fluorogold labeled neurons in the NTS. Only animals with histologically confirmed AP-lesion and similar numbers of fluorogold positive NTS neurons as sham lesioned animals were included for the final statistical analysis.

*Capsaicin vagotomy*

The local vagal capsaicin treatment was performed as previously described (Raybould and Tache, 1988). Surgery success and specificity was verified functionally and histologically. The functional test was the absence of CCK induced satiation, as described above. Capsaicin treatment was considered successful if CCK reduced eating 30 min post application by less than 30% compared to controls (3 of 13 animals excluded; food intake: sham NaCl 4.8 ± 0.2 g, sham CCK 2.5 ± 0.3 g, Capsaicin-treated NaCl 3.9 ± 0.g g, Capsaicin-treated CCK 3.7 ± 0.3 g). Histological validation of the surgery included a test for the anterograde labeling capacity of vagal afferents to the NTS as described above. Only animals with histologically confirmed fluorogold staining in the AP (as injection control) and decreased fluorogold staining in the NTS (due to damaged vagal afferents) were included into the final statistical analysis.

*Conditioned taste aversion*

Water access is restricted during the 14 days of the conditional taste aversion test. Importantly animals received access to water or saccharine specific to the experimental day as specified

below for a 30 min period at dark onset. In the first 4 days of the experiment rats received access to two water bottles to adapt animals to the drinking schedule. On day 5 rats received access to two bottles saccharine (0.3 % in tap water, Sigma). Saccharine intake was equal between experimental groups on day 5 thereby showing equal preference for saccharine intake. Following the 30 min access to saccharine, amino acids (IG, 6.7 mmol/kg; IV 2 mmol/kg) or LiCl (IP, 76 mg/kg, Sigma), which was used as positive control, were administered. This conditioning paradigm was repeated on days 8 and 11; in between, i.e. on days 6-7, 9-10 and 12-13, respectively, water was offered as described for days 1-4. On Day 14 rats were presented one bottle of water and one bottle of 0.3 % saccharine for 30 min at dark onset. Rats were offered water 4 h after dark onset for a 45 min period every day to ensure proper hydration. Liquid intake was measured throughout the entire experiment by weighting the bottles.

*Images*

Stomach images were captured with a digital camera (D50, Canon), brain slice images with the microscope (Axio Imager 2, Zeiss). Images were not altered except for minor adjustments in brightness and contrast. Fluorogold images were black/white inverted for better visibility.

*Statistics*

Rats were randomly allocated into treatment and/or surgical group. Application order was randomized. Results are presented as mean ± standard error of the mean. The data were analyzed using Graph Pad Prism 5.0.

**Results:**

Identification of the most potent oral anorectic amino acids

To identify the most potent anorectic individual amino acid, we administered isomolar doses of all 20 proteogenic amino acids individually to rats via the physiological gastrointestinal route and measured subsequent food intake. The amino acids were administered by gavage at an equal dose of 6.7 mmol/kg, corresponding in the case of Glu to 1g/kg and 27% of average daily Glu intake. Surprisingly the anorectic response induced by individual amino acids did not follow a rank order according to side chain structure, energy content, nutritional necessity (essential) or role as neurotransmitter (-precursor), but rather revealed a unique role for Arg, Lys and Glu (Fig. 1A). Each of these three amino acids significantly reduced food intake in the first hour after administration, whereas all other amino acids had no significant effect compared to water control (Fig. 1A). In the long term only Arg and Lys decreased food intake, whereas Glu treated animals compensated for the initial decrease in food intake in the following 48 h (Fig. 1B). Consistent with previous studies, oral Trp had a small effect on food intake which, however, was statistically not significant when testing for multiple comparisons (Fig. 1A, unpaired two-tailed t-test, p=0.03, Ng and Anderson, 1992); the other aromatic amino acids Phe or Tyr did not inhibit food intake as previously shown (Bialik et al. 1989). Meal pattern analysis indicated that oral Arg, Lys and Glu administration reduced eating only by an effect on the first meal size while the latency to meal initiation, meal number, meal duration or time between meals did not differ (Fig. 1C-F). Additional control experiments excluded that pH, osmolarity or the feeding state, hence the presence of nutrients, altered the anorectic effect of Lys (Fig.S1); dose response studies indicated a plateauing of the anorectic effect at 4.7 to 5.4 mmol/kg (Fig. S2A-C). Drinking behavior was not altered by Arg and Lys, but Glu reduced water intake in a dose dependent

manner (Fig. S2D-F). These findings are striking because they identify Arg, Lys and Glu as the individual amino acids with the strongest anorectic effect.

Neuronal pathways mediating the anorectic effect of Arg, Lys and Glu

Eating behavior is ultimately controlled by the brain, which integrates a large array of sensory inputs projecting to specific areas (Berthoud, 2002). Therefore we investigated where in the brain Arg, Lys and Glu triggered neuronal activity by assessing the number of cells expressing cFOS, a neuronal activity marker induced by increases in intracellular $Ca^{2+}$ (Watts et al. 2006). Upon gavage with these three amino acids, an increased number of cFOS positive cells were detected in the nucleus of the solitary tract (NTS) and the area postrema (AP); two areas known to mediate the effect of specific anorectic signals (Fig. 2). Importantly, the AP is not protected by the blood brain barrier such that it may directly sense blood borne signals, and the NTS is the main projection site of vagal afferents (Morton et al. 2006). In contrast, the nucleus accumbens or the hypothalamus (arcuate nucleus, paraventricular nucleus of the hypothalamus, lateral hypothalamic area) did not display increased cFOS expression at this time point. To discriminate whether the anorectic effect of Arg, Lys and Glu was mediated via the AP or vagal afferents, we surgically lesioned both routes. The AP was vacuum aspirated, whereas vagal afferents were chemically lesioned by local sub-diaphragmatic capsaicin application. Surgery specificity and success was validated functionally and histologically (Fig. 3A). Arg and Glu lost their anorectic effect in AP-lesioned animals, but not in capsaicin-treated animals (Fig. 3B-C). Lys, on the other hand, showed an opposite response to the surgical interventions because its anorectic effect was dependent on intact vagal afferents but not on intact AP (Fig. 3). These findings suggest that the anorectic response to Arg and Glu is induced centrally in the area postrema, whereas Lys response is relayed from the periphery to the NTS by abdominal vagal afferents.

Satiation effects are dissociated from gastrointestinal actions

Vagal afferents fire also in response to gastric distension, which may contribute to meal-ending satiation (Woods, 2009). Apart from the ingestion of food and water, gastric distension can also be due to gastric secretion or delayed gastric emptying, the latter described to depend mainly on the gastric caloric content (Camilleri, 2006). Arg, Lys and Glu increased gastric distension 0.5 h after their application, an effect which coincided with the time of decreased food intake (Fig. 4A). The increase in gastric volume by Arg and Lys administration appeared to be mediated by acidic gastric secretion as the alkaline tide increased plasma albumin-, and decreased plasma $Cl^-$ concentration (Tab. S1). Gastric emptying was delayed by Lys and Glu, as measured by phenol red retention in the stomach (Fig. S3A). For all three amino acids, wet weight of the stomach and/or cecum was still increased 1.5 h after amino acid administration (Fig. 4B). These findings demonstrate that specific amino acids differentially modulate gastrointestinal function, an important finding in light of the caloric focus in this field (Camilleri, 2006). We next tested whether the gastric and anorectic responses to Arg, Lys and Glu are functionally linked by bypassing the gastrointestinal lumen through intravenous amino acid administration. The dose of 2 mmol/kg was selected, because it led to similar changes in plasma amino acid concentration as the oral dose (6.7 mmol/kg) given by gastric gavage (Tab. S2). Intravenous Arg, Lys and Glu reduced food intake similar as after oral application, but only intravenous Glu had an effect on gastric function (Fig. 5, Fig. S3B, Tab. S2). However, in AP-lesioned animals Glu delayed gastric emptying, but did not inhibit food intake (Fig. 3; Fig. S4). These data suggest that all three amino acids Arg, Lys and Glu, inhibit food intake through circulation and that their effect on gastrointestinal function can be dissociated from their anorectic effect.

Finally we tested if the three amino acids cause visceral discomfort. Therefore we conducted a conditioned taste aversion test after Arg, Lys and Glu administration by the intragastric respectively intravenous route. Oral Glu treatment did not alter the innate preference to saccharine of rats, whereas oral Arg and Lys did induce conditioned taste aversion (Fig. 6A). If Arg and Lys were given intravenously animals did not reduce their saccharine intake (Fig. 6B). These data suggests that the gastrointestinal function changes caused by oral Arg and Lys, but not by intravenous Arg and Lys, induced conditioned taste aversion. Importantly the anorectic effect of Arg and Lys was equally prominent after oral or intravenous application suggesting limited importance of visceral discomfort for their anorectic effect.

**Discussion**:

Our study establishes a novel role of the amino acids Arg, Lys and Glu in the control of food intake and gastrointestinal function. This expands the existing concept of gastrointestinal function modulation through the caloric content of meals by adding specific amino acids as novel calorie-independent signals (Camilleri, 2006). By inducing gastric secretion these amino acids may favor acid-mediated denaturation of proteins and, by delaying gastric emptying, extend the time for enzymatic protein digestion. Hence, we suggest that the gastrointestinal tract recruits appropriate digestive capacity to cope with a high protein load based on specific amino acid detection. Future work should more systematically test the effect of individual amino acids on gastrointestinal function with an emphasis on translating animal findings to humans. Gastric distension is a mechanical stimulus, transmitted by vagal afferent to the NTS that may contribute to the control of food intake (Schwartz, 2006; Woods, 2009; Davis et al. 1993).Our experiments did however not support such a nutrient unspecific gut-brain axis and showed a clear dissociation of the gastric and the behavioral responses to Arg, Lys and Glu as schematically represented in Fig. 7. In agreement, gastric distensions in balloon inflation studies did not inhibit food intake in rats and humans (Oesch et al. 2006).

Our data highlight the importance of circulating amino acids for the control of food intake and we identified Arg, Lys and Glu as the most effective ones, at least at isomolar doses. Naturally a complete dose-response relationship for all twenty amino acids would be desirable, nevertheless the here used single dose approach enabled the identification of the most potent anorectic amino acids. All three induced satiation by reducing first meal size, but not meal duration, unlike classical satiation-inducing peptides such as CCK or amylin which proportionally reduce meal

size and duration (Woods, 2009). Interestingly, the observed amino acid specificity is in marked contrast to the previously emphasized unique role of the amino acid Leu, which inhibits food intake by regulating mTOR signaling when administered directly to the third brain ventricle (Cota et al. 2006; Morrison et al. 2007). Indeed, consistent with others who showed that physiological changes in plasma Leu concentration do not lead to a reduction of food intake, our data also do not support a role of peripheral Leu for food intake control (Laviano et al. 2006; Zhang et al. 2007; Purpera et al. 2012; Nassl et al. 2011; Morrison et al. 2012). Importantly, so far no systematic study on the effect of the 20 proteogenic amino acids had been published (Fig. 1A). Here, we unravel an amino acid specificity that can be interpreted either as an evolutionary reductive strategy to detect the few amino acids that most accurately indicated protein supply or as a strategy to detect protein quality (Lamb, 2012). Supporting the latter possibility, high protein diets of different sources differentially alter satiation in rodents and humans (Faipoux et al. 2006; Anderson et al. 2004; Pichon et al. 2008).

Interestingly two different afferent pathways respond specifically to one of the candidate amino acids. This redundancy may ensure accurate detection of protein intake *in vivo*. Others have already proposed the existence of a Lys sensor located on vagal afferents innervating the hepatic portal vein. However, the molecular identity of the sensor remained obscure (Torii K & Niijima, 2001). Similarly the molecular mechanism of Arg and Glu sensing by area postrema neurons is unknown, but other previously reported neurons able to sense specific amino acids (Cota et al. 2006; Blouet & Schwartz, 2012; Karnani et al, 2011). We identified here three novel amino acids as potent modulator of short-term food intake and suggest their neuronal site of action. They can contribute to the anorectic effect of a high protein diet as Arg, Lys and Glu plasma concentrations were shown to raise proportionally to the protein content of a meal (Peters &

Harper, 1987). Analogously dietary Arg supplementation was shown to reduce body weight gain in rats (Fu et al, 2005). Based on the aversive response rat exhibit to diets depleted from essential amino acids, we expect a diet depleted from Arg to be rejected but we are currently not aware of such a study (Morrison et al, 2012). One can speculate that a diet supplemented or partially restriction of the three candidate amino acids may mimic the effect of a high respectively of a low protein diet on feeding behavior. Taken together we propose that the central nervous system detects dietary protein content and quality to control food intake by sensing specific circulating amino acids, in particularly Arg and Glu via the AP and Lys via vagal afferents (Fig. 7).

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**Figure Legends:**

**Figure 1. Intragastric Arg, Lys and Glu inhibited food intake most potently of all proteogenic amino acids.** After 12 h food deprivation rats were gavaged with isomolar doses of individual amino acids (6.7 mmol/kg, which in the case of Glu corresponds to 1 g/kg and to 27 % of the daily average Glu intake) and their subsequent food intake measured for 1 (A), 2, 4, 24 and 48 h (B); n = 12, mean ± sem; (A) unpaired one-way ANOVA, Dunnett post test; (B) unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001. (C-F) After 16 h food deprivation rats were gavaged with individual amino acids (6.7 mmol/kg) and their subsequent meal pattern analyzed in an automated BioDaq system; n = 10, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05.

**Figure 2; Intragastric Arg, Lys and Glu induced neuronal activity in the brainstem.** After 16 h food deprivation, rats were gavaged with isomolar doses (6.7 mmol/kg) of individual amino acids and transcardially perfused with PFA 2 h later. Animals had no access to food between gavage and transcardial perfusion. Different brain areas were analyzed for cFOS expression. (A) Representative images showing cFOS positive cells in the AP and the NTS (located -13.76 from bregma). Scale bar, 100 μm. AP indicates the area postrema, NTS – nucleus of the solitary tract, 4V – 4th ventricle, DMX – dorsal motor nucleus vagus nerve, GR – gracile nucleus. Quantification of cFOS positive cells in the AP (B) and NTS (C); n = 6-8, mean ± sem; unpaired one-way ANOVA, Dunnett post test; **p < 0.01, ***p < 0.001.

**Figure 3. The AP mediated the anorectic effect of Arg and Glu, whereas abdominal vagal afferents are necessary for the anorectic effect of Lys.** (A) Histological surgery validation showed complete removal of the AP and an intact vagus nerve, visualized by retrograded tracing of IP injected fluorogold to the NTS (primary vagal projection site), in AP-lesioned animals.

Capsaicin treatment lesioned the vagus nerve and consequently less fluorogold reached the NTS in respective animals. The AP showed fluorogold labeling due to the absence of the blood brain barrier. Location -13.76 from bregma, 4V indicates the 4th ventricle, DMX – dorsal motor nucleus vagus nerve, GR – gracile nucleus, scale bar 100 μm. After 16 h food deprivation AP-lesioned (B), capsaicin-treated (C) and the respective sham animals were gavaged with individual amino acids (6.7 mmol/kg) and their subsequent food intake measured for 1 h; n = 7-11, mean ± sem; repeated measures two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001.

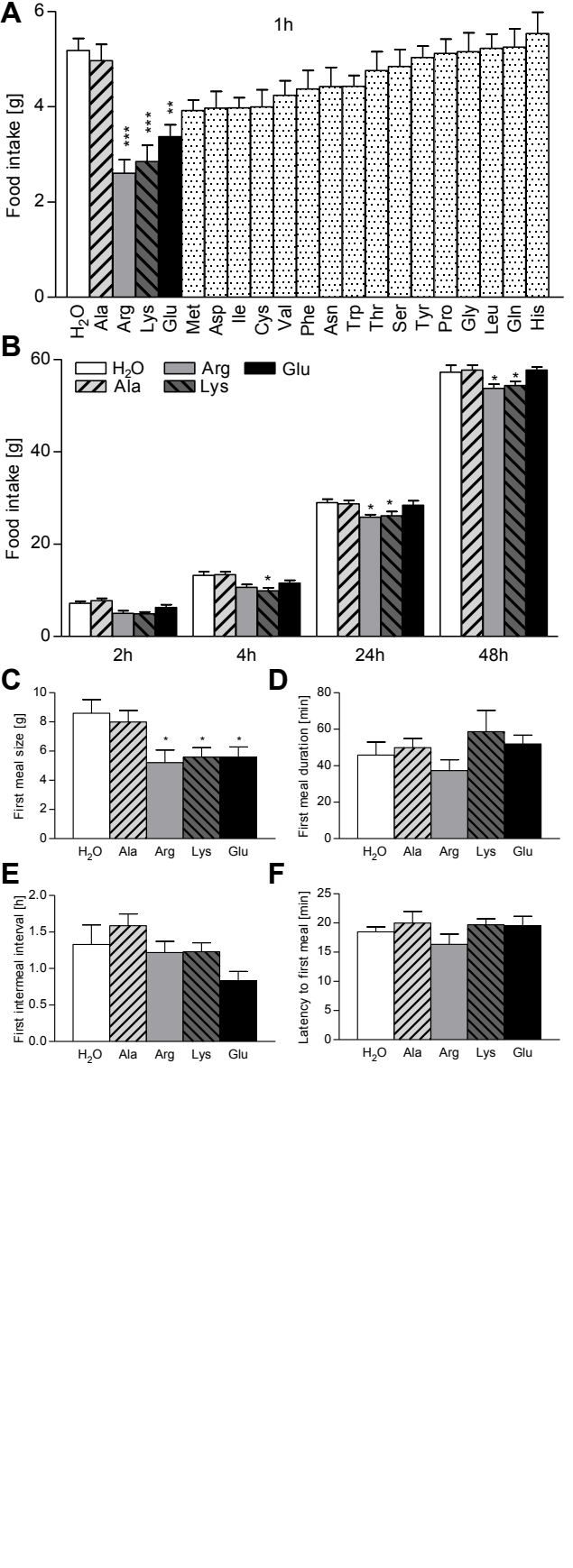
**Figure 4. Arg, Lys and Glu induced gastric distension by distinct mechanisms.** (A) After 16 h food deprivation rats were gavaged with an isovolumic (2 ml) dose of individual amino acids (6.7 mmol/kg), 30 min later their stomach was excised and weighted; n = 6, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, ***p < 0.001. Representative images are shown; scale bar, 4 mm. (B) After 16 h food deprivation rats were gavaged with individual amino acids (6.7 mmol/kg), 30 min later received access to 3 g rat chow and 1.5 h post-administration the gastrointestinal tract was excised and the wet weight measured; n = 6, mean ± sem; unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001.

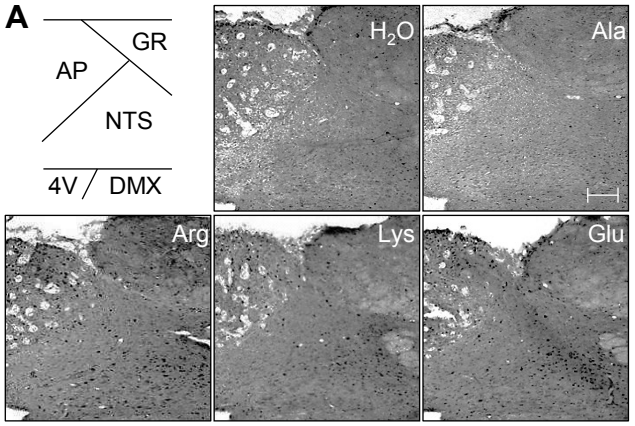
**Figure 5. Intravenous Arg, Lys and Glu inhibited food intake, but only intravenous Glu delayed gastric emptying.** (A) After 16 h food deprivation rats were anesthetized, received a Ringer lactate (RLA) or amino acid (2 mmol/kg) injection into the lateral tail vein, were gavaged with water or amino acid (HK, 6.7 mmol/kg Lys; LK, 2 mmol/kg Lys) and their subsequent food intake was measured for 1 h; n = 9-11, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, **p < 0.01, ***p < 0.001. (B) After 16 h food deprivation rats were anesthetized, received a Ringer lactate (RLA) or amino acid (2 mmol/kg) injection into the

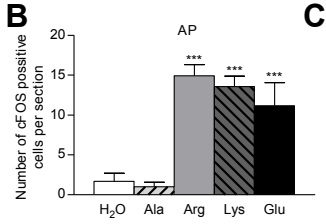
lateral tail vein, were gavaged with water or amino acid (HK, 6.7 mmol/kg Lys; LK, 2 mmol/kg Lys), 30 min later their stomach was excised and weighted; n = 6, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, ***p < 0.001. (C) After 16 h food deprivation rats were anesthetized, received a Ringer lactate (RLA) or amino acid (2 mmol/kg) injection into the lateral tail vein, were gavaged with water, 30 min later received access to 3 g rat chow and 1.5 h post-administration the gastrointestinal tract was excised and the wet weight measured; n = 6, mean ± sem; unpaired two-way ANOVA, Bonferroni post test; *p < 0.05.

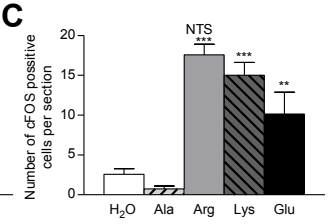
**Figure 6. Conditioned taste aversion test following different routes of amino acid administration.** (A) Rats received an amino acid (6.7 mmol/kg) or water gavage during the conditioning days. (B) Rats received an amino acid (2 mmol/kg) or Ringer lactate (RLA) injection into the lateral tail vein and were gavaged with water or amino acid (HK, 6.7 mmol/kg Lys) during the conditioning days. IP inject LiCl is the positive control. n = 6 (LiCl n = 3), mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, **p < 0.01, ***p < 0.001.

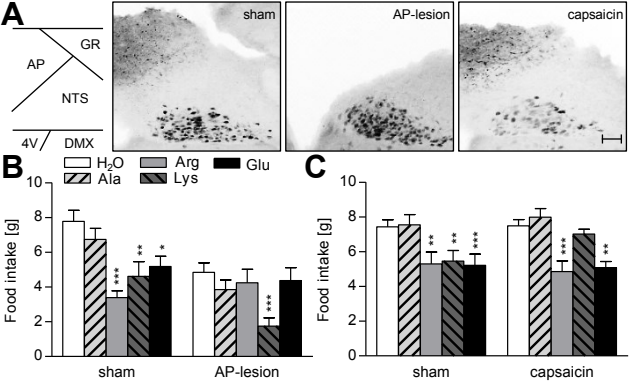
**Figure 7. Schematic representation of main findings.** Arg, Lys and Glu were identified as the most potent oral anorectic amino acids. After gastric delivery, they specifically stimulate gastric secretion and/or delay gastric emptying thereby facilitating digestion. After absorption only Glu can inhibit gastric emptying. Importantly all three amino acid also inhibit food intake when administered intravenously but by different neuronal mechanism. Lys is detect by vagal afferents projecting to the NTS, whereas Arg and Glu centrally in the area postrema - a brain areas not protected by the blood barrier. Thus, Arg, Lys and Glu selectively impact on food processing and intake suggesting them as direct sensory input to assess dietary protein content and quality.


A
1h
Food intake [g]
0
2
4
6
***
***
**
H2O
Ala
Arg
Lys
Glu
Met
Asp
Ile
Cys
Val
Phe
Asn
Trp
Thr
Ser
Tyr
Pro
Gly
Leu
Gln
His
B
H2O
Ala
Arg
Lys
Glu
Food intake [g]
0
20
40
60
2h
4h
24h
48h
C
First meal size [g]
0
2
4
6
8
10
H2O
Ala
Arg
Lys
Glu
D
First meal duration [min]
0
20
40
60
80
H2O
Ala
Arg
Lys
Glu
E
First intermeal interval [h]
0.0
0.5
1.0
1.5
2.0
H2O
Ala
Arg
Lys
Glu
F
Latency to first meal [min]
0
5
10
15
20
25
H2O
Ala
Arg
Lys
Glu


A
GR
AP
NTS
4V
DMX
H2O
Ala
Arg
Lys
Glu

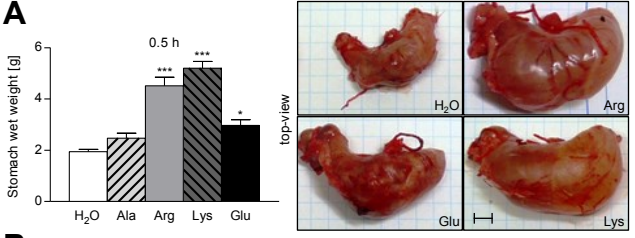

B
AP
Number of cFOS possitve cells per section
20
15
10
5
0
***
***
***
H2O
Ala
Arg
Lys
Glu

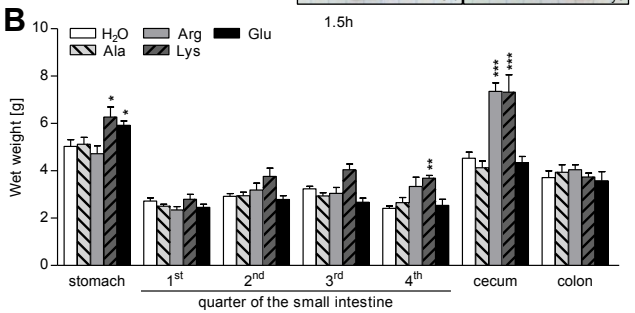

C
NTS
Number of cFOS possitve cells per section
20
15
10
5
0
***
***
**
H2O
Ala
Arg
Lys
Glu

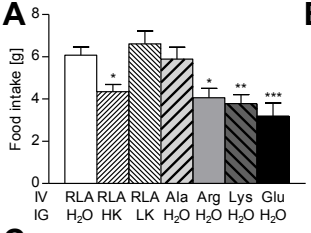

A
GR
AP
NTS
4V
DMX
sham
AP-lesion
capsaicin

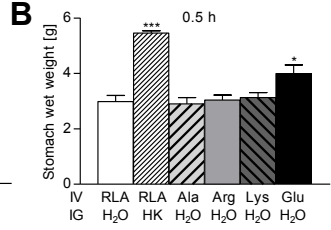

B
H2O
Ala
Arg
Lys
Glu
Food intake [g]
0
2
4
6
8
10
***
**
*
***
sham
AP-lesion

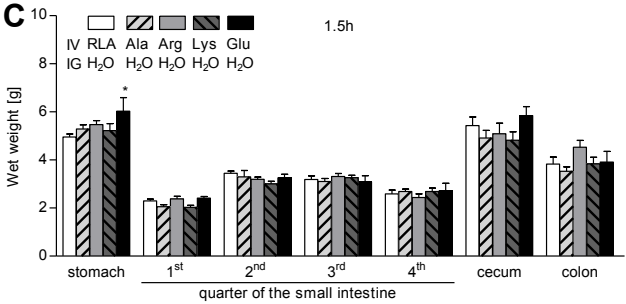

C
Food intake [g]
0
2
4
6
8
10
**
**
***
***
**
sham
capsaicin

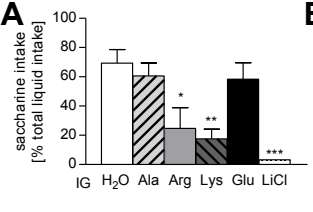

A
0.5 h
Stomach wet weight [g]
6
4
2
0
***
***
*
H2O
Ala
Arg
Lys
Glu
top-view
H2O
Arg
Glu
Lys

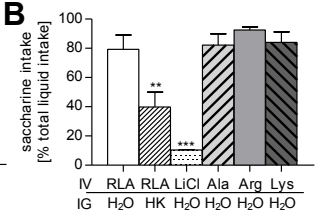

B
1.5h
H2O
Ala
Arg
Lys
Glu
Wet weight [g]
10
8
6
4
2
0
*
*
**
***
***
stomach
1st
2nd
3rd
4th
cecum
colon
quarter of the small intestine

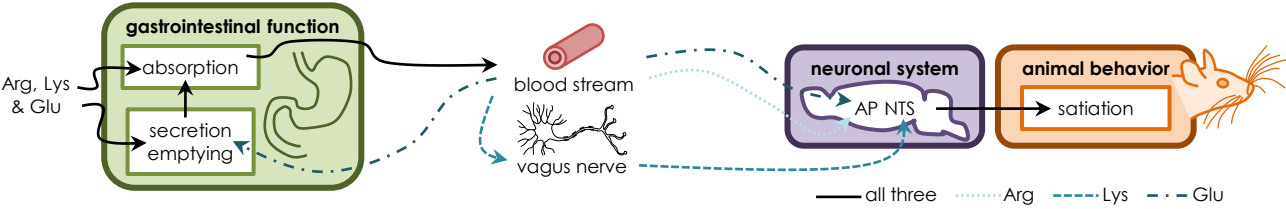

A
Food intake [g]
0
2
4
6
8
*
*
**
***
IV RLA RLA RLA Ala Arg Lys Glu
IG H2O HK LK H2O H2O H2O H2O


B
0.5 h
Stomach wet weight [g]
0
2
4
6
***
*
IV RLA RLA Ala Arg Lys Glu
IG H2O HK H2O H2O H2O H2O


C
1.5h
IV RLA Ala Arg Lys Glu
IG H2O H2O H2O H2O H2O
Wet weight [g]
0
2
4
6
8
10
*
stomach
1st
2nd
3rd
4th
quarter of the small intestine
cecum
colon


A
saccharine intake
[% total liquid intake]
100
80
60
40
20
0
*
**
***
IG
H2O
Ala
Arg
Lys
Glu
LiCl


B
saccharine intake
[% total liquid intake]
100
80
60
40
20
0
**
***
IV
RLA
RLA
LiCl
Ala
Arg
Lys
IG
H2O
HK
H2O
H2O
H2O
H2O


gastrointestinal function
absorption
secretion
emptying
Arg, Lys
& Glu
blood stream
vagus nerve
neuronal system
AP NTS
animal behavior
satiation
all three
Arg
Lys
Glu